J. Membrane Biol. 87, 77–91 (1985)

The Journal of
Membrane Biology
© Springer-Verlag 1985

Topical Review

The Purification of Ion Channels from Excitable Cells

Jane A. Talvenheimo*
Department of Pharmacology, University of Washington, Seattle, Washington 98195

Introduction

Ion channels are the membrane proteins underlying the transmission of electrical signals and the transduction of neurotransmitter signals in electrically excitable cells. They can be divided into two major classes: ligand-gated channels, such as the nicotinic acetylcholine receptor, which respond to chemical signals, and voltage-gated channels, such as the voltage-sensitive sodium channel, which respond to changes in membrane potential.

Since Hodgkin and Huxley [71] first described the membrane conductance changes that accompany the action potential in squid giant axons, every known ion channel has been characterized initially by a description of its electrical properties. Direct electrical recording of membrane conductance changes in response to chemical or electrical stimuli is still the primary technique for discovering and studying the function of ion channels. With the development of more sophisticated recording techniques, in particular the gigaohm-seal patch-clamp method, the number of ion channels that have been described has grown rapidly.

In addition to the voltage-dependent sodium and potassium channels first identified in squid axon, at least three other voltage-dependent potassium channels have been described: the inward rectifier potassium channel, the calcium-activated potassium channel, and the fast "A channel." Voltage-dependent chloride channels, calcium-dependent chloride channels, and cation channels associated with muscarinic acetylcholine receptors have also been described. The most extensively studied receptor-controlled ion channel is the nicotinic acetylcholine receptor-associated channel, but the number of receptor-associated channels that have been identified is growing and includes ion channels controlled by gamma aminobutyric acid (GABA)[1], glutamate, and glycine receptors. Hille [70] has presented a comprehensive description of the biophysical properties and functions of these major classes of ion channel.

When the sodium and potassium permeability changes that occur during an action potential were first studied, the physical basis for these membrane permeability changes was completely unknown. The concept that ion channels are transmembrane proteins capable of undergoing conformational changes, resulting in the opening or closing of an aqueous pore, is relatively recent. Although it is now accepted that ion channel function is, in many ways, analogous to enzyme function, detailed biochemical information on channel structure is available for only a few channels. The primary amino-acid sequences are known only for the nicotinic acetylcholine receptor [35, 43, 104, 106–108, 133] and the voltage-sensitive sodium channel [105]. The subunit composition of the purified GABA receptor has been determined [13, 131], but is has not been conclusively shown that the purified receptor contains the associated chloride channel. The calcium antagonist receptor associated with the voltage-dependent calcium channel has been partially purified [20, 39, 56], but it is not yet known whether the polypeptides identified as part of the drug receptor site on the channel actually comprise the entire calcium channel. For most ion channels, even those for which detailed electrophysiological data have been obtained, relatively little biochemical information is available.

---

* *Present address:* Department of Pharmacology, University of Miami School of Medicine, P.O. Box 016189, Miami, Florida 33101

[1] *Abbreviations:* GABA, gamma aminobutyric acid; TTX, tetrodotoxin; STX, saxitoxin; LqTx, *Leiurus quinquestriatus* toxin; DEAE, diethylaminoethyl; T-tubule, transverse tubule; SDS, sodium dodecyl sulfate; CHAPS, 3-[(3-cholamidopropyl)dimethyl-ammonio]-1-propanesulfonic acid.

Table 1. Neurotoxin receptor sites on the sodium channel

Site	Neurotoxins	Physiological effect
1	Tetrodotoxin Saxitoxin	Inhibit ion transport
2	Alkaloid toxins: Veratridine Batrachotoxin Grayanotoxin Aconitine	Cause sodium channels to be persistently activated
3	North African α scorpion toxins: *Leiurus quinquestriatus* *Androctonus australis*	Inhibit inactivation and enhance the action of alkaloid toxins
4	American β scorpion toxins: *Centruroides suffusus* *Tityus serrulatus*	Enhance activation

The purpose of this review is threefold: first, to describe the approach used to purify the voltage-sensitive sodium channel and to compare the structure of the channel isolated from three different sources; second, to summarize the recent progress in isolating the voltage-dependent calcium channel; and third, to describe briefly the genetic approaches that are being used to identify the polypeptide components of potassium channels. It is appropriate to mention that the nicotinic acetylcholine receptor was the first receptor/ion channel complex to be purified to homogeneity, reconstituted in a completely functional form, and sequenced by identifying and sequencing the DNA coding for the receptor polypeptides. The biochemical techniques developed to study the acetylcholine receptor have facilitated the development of methods for purifying other ion channels. However, since these results have already been extensively reviewed [8, 36, 81, 89, 103, 112], the biochemistry of the acetylcholine receptor will not be included in this review.

Key Words ion channels · excitable membranes · sodium channel · calcium channel · reconstitution

Neurotoxin Probes for the Voltage-Dependent Sodium Channel

Identification of the sodium channel polypeptides in intact cells and in membrane preparations has been made possible by the existence of four major classes of neurotoxin (Table 1) that interact specifically with four separate sites on the channel [for reviews *see* 1, 29, 30, 101]. The first group of toxins, tetrodotoxin (TTX) and saxitoxin (STX), bind to an external site on the sodium channel and completely block ion movement through the channel [69, 85, 100, 102]. Both of these toxins can be labeled to a high specific activity with tritium [115]. Labeled STX and TTX bind competitively to a single high-affinity site on the sodium channel, with dissociation constants in the nanomolar range. Because TTX and STX bind to the channel very selectively, they have been particularly useful for isolating the sodium channel from membrane preparations.

The second class of toxins that interact with the sodium channel are the alkaloid toxins, a group of lipid-soluble compounds, including veratridine and batrachotoxin, that bind to a second site on the sodium channel. The alkaloid toxins block sodium channel inactivation and shift the voltage dependence of activation, allowing the channel to activate at the resting potential and remain in the activated state even after prolonged depolarization [6, 147]. These toxins provide a biochemical means for activating the sodium channel at the resting membrane potential. Using the alkaloid toxins to activate sodium channels, assays have been developed to measure $^{22}Na^+$ fluxes through the channel [25, 26, 32]. This has been a valuable technique for studying sodium channel function in preparations that cannot be voltage-clamped, such as synaptosomes [138].

The third major class of toxins are the polypeptide toxins isolated from North African scorpion venoms (alpha scorpion toxins) and from sea anemones. These toxins bind to a third site on the channel, blocking inactivation [116–118]. A toxin isolated from the scorpion *Leiurus quinquestriatus* (LqTx), which can be labeled with ^{125}I [27], binds to the channel in a voltage-dependent manner [27, 28, 33]. LqTx binding to the sodium channel provides a sensitive biochemical probe for voltage-dependent conformational changes in channel structure.

The beta scorpion toxins, isolated from American scorpion venoms, comprise the fourth major class of neurotoxin. These toxins bind to a fourth site on the sodium channel, distinct from the alpha scorpion toxin binding site, and alter the activation kinetics of the channel [11, 22, 37, 150]. Beta scorpion toxins have been used in photoaffinity labeling experiments [41] to identify sodium channel polypeptides, and as probes for the solubilized sodium channel [12, 110].

Identification of Rat Brain Sodium Channel Polypeptides by Photoaffinity Labeling with a Photoreactive Scorpion Toxin Derivative

The first approach used to identify the sodium channel subunits from rat brain was to prepare a photoreactive derivative of the alpha scorpion toxin

LqTx [15]. ^{125}I-labeled azidonitrobenzoyl-LqTx labels two polypeptides in rat brain synaptosomes, one with a molecular weight of 260,000 and a smaller polypeptide with a molecular weight of about 32,000 (Fig. 1). The covalent incorporation of ^{125}I-azidonitrobenzoyl-LqTx into both of these membrane polypeptides was blocked when the labeling procedure was carried out in the presence of excess unlabeled LqTx or sea anemone toxin. Labeling was also blocked by depolarizing the synaptosomes, which blocks high-affinity binding of LqTx to the sodium channel. This experiment provided the first evidence that two polypeptides are closely associated with the alpha scorpion toxin binding site on the mammalian brain sodium channel. However, since LqTx does not bind to the sodium channel in disrupted or solubilized membrane preparations [31], its usefulness as a ligand for purifying the sodium channel is limited.

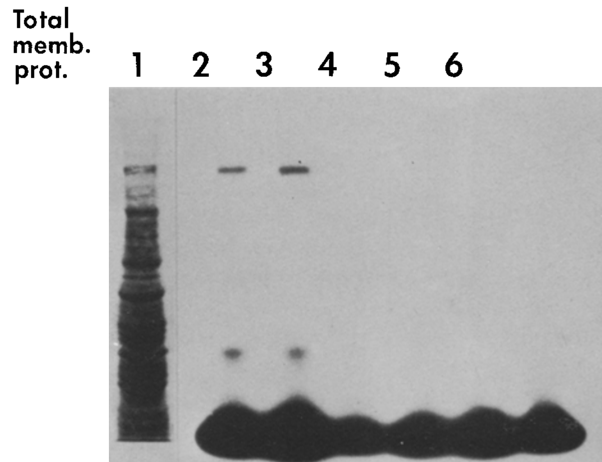

Fig. 1. Labeling of sodium channel polypeptides by a photoreactive derivative of scorpion toxin. Intact synaptosomes were incubated with 2 nM ^{125}I-azidonitrobenzoyl-LqTx, which was activated by irradiation with ultraviolet light. The labeling was carried out in the presence of 1 μM TTX (Lane 1); 1 μM TTX and 1 μM batrachotoxin (Lane 2); 1 μM TTX and 200 nM unlabeled LqTx (Lane 3); 1 μM TTX, 1 μM batrachotoxin, and 200 nM unlabeled LqTx (Lane 4); 135 mM KCl, 1 μM TTX, and 1 μM batrachotoxin (Lane 5); and 135 mM KCl, 1 μM TTX, 1 μM batrachotoxin, and 200 nM unlabeled LqTx (Lane 6). Samples were analyzed by SDS polyacrylamide gel electrophoresis and autoradiography. Total synaptosomal membrane proteins, stained with Coomassie blue, are shown in the far left. (Reprinted from Beneski and Catterall [15])

Solubilization and Stabilization of the Voltage-Sensitive Sodium Channel

Henderson and Wang [68], followed by Benzer and Raftery [16], were the first to show that TTX binding activity is retained when axonal membranes are solubilized with a nonionic detergent. Although it was immediately recognized that ^{3}H-TTX could be used as a probe for the detergent-solubilized sodium channel during purification procedures, the instability of the solubilized binding activity proved to be a difficult obstacle to initial purification attempts. Conditions for stabilizing the solubilized TTX binding protein were first established by Agnew and Raftery [4]. They found that the TTX binding component of the electric eel sodium channel, solubilized in Lubrol-PX, is stabilized by the addition of exogenous phospholipid. It was subsequently shown that the stability of the solubilized TTX binding activity from rat brain [62] and rat skeletal muscle [10] also depends on the addition of exogenous lipid, and can be improved by including Ca^{2+}.

Purification and Subunit Composition of the Sodium Channel from Rat Brain

Hartshorne and Catterall [62, 63], using conditions to optimize the stability of STX binding activity in Triton X-100 extracts, developed a procedure for purifying the sodium channel from rat brain. The solubilized STX receptor has a molecular weight of 316,000, determined by sucrose gradient sedimentation [64]. Assuming one mole of STX binds to one mole of sodium channel, the maximum theoretical specific STX binding activity for pure sodium channel is 3200 pmol STX bound per mg of membrane protein, corresponding to a 1000-fold purification from rat brain membranes. Specific activities of 2910 pmol STX bound per mg protein, representing a preparation that is greater than 90% pure, are obtained by purifying the solubilized STX binding activity through four steps: sequential chromatography on DEAE-Sephadex, hydroxylapatite, and wheat germ agglutinin-Sepharose, followed by sucrose gradient sedimentation [63].

Three polypeptide subunits, α (mol wt = 260,000), $\beta 1$ (mol wt = 39,000), and $\beta 2$ (mol wt = 37,000), comigrate with STX binding activity throughout this procedure. On silver-stained SDS-polyacrylamide gels, α, $\beta 1$, and $\beta 2$ account for more than 90% of the total staining intensity. The subunit composition of the purified rat brain sodium channel is shown in Fig. 2. The smaller of the two β subunits, $\beta 2$, is covalently attached to the α subunit by disulfide bonds [67]. The $\beta 1$ subunit, which is associated with the α subunit by noncovalent interactions, corresponds to the smaller of the two polypeptides specifically labeled by photoreactive derivatives of LqTx [15, 67, 130]. A polypeptide with a

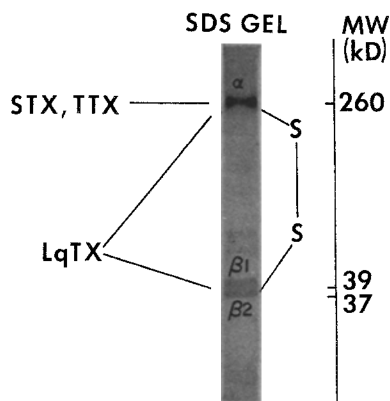

Fig. 2. Subunit composition of the purified rat brain sodium channel. Purified sodium channel from a sucrose gradient fraction was analyzed by SDS polyacrylamide gel electrophoresis. The alpha and beta subunits were visualized by silver staining

molecular weight identical to that of β1 is also labeled by a photoreactive derivative of *Centruroides suffusus* scorpion toxin [41].

Barhanin et al. [12], using a purification procedure almost identical to that of Hartshorne and Catterall [62], isolated the rat brain sodium channel by prelabeling it with *Tityus serrulatus* toxin. *Tityus serrulatus* toxin is a beta scorpion toxin that binds to a site distinct from the LqTx binding site. A single large polypeptide (mol wt = 270,000) comigrated with *Tityus serrulatus* scorpion toxin binding activity. This polypeptide is presumably identical to the large α subunit covalently labeled by ^{125}I-azidonitrobenzoyl-LqTx [15] and subsequently identified by Hartshorne and Catterall [62, 63] as part of the purified STX receptor. In partially purified preparations from rat brain, Barhanin et al. [12] did not observe any subunits corresponding to the β1 and β2 subunits identified by Hartshorne and Catterall. The reason for this discrepancy has not yet been established.

Polypeptide Composition of the Sodium Channel Isolated from Rat Skeletal Muscle

Barchi et al. [10] have solubilized the STX binding site from rat sarcolemmal membranes with Nonidet P-40. The STX binding activity was purified by ion exchange chromatography on a guanidinium resin, followed by chromatography on wheat germ agglutinin-Sepharose. In the initial study the sarcolemmal sodium channel was reported to consist of three peptides with molecular weights of 53,000, 60,000 and 64,000. However, in subsequent studies, sucrose gradient sedimentation has been included as the final step in the purification [9, 151], and proteolysis has been limited by the inclusion of protease inhibitors throughout the procedure. In the more recent studies, the purified sarcolemmal sodium channel, which has a specific activity of 2500 pmol STX bound per mg of protein, is reported to contain a large subunit with a molecular weight of 260,000, a smaller subunit with a molecular weight of 45,000, and a doublet at 38,000 [9, 24].

Polypeptide Composition of the Electric Eel Sodium Channel

The electric organ of *Electrophorus electricus* contains a high density of sodium channels, providing an excellent source of sodium channels for purification. Using three purification steps, ion exchange chromatography followed by two gel filtration steps, Agnew et al. [2] reported substantial purification of the TTX binding component solubilized from eel electroplax with the nonionic detergent Lubrol-PX. Of the three peptides that consistently appeared in this partially purified preparation, only a single large polypeptide with a molecular weight of 260,000 comigrated with TTX binding activity [3]. This molecular weight was consistent with a size of 230,000 for the TTX binding site, which had been estimated from radiation inactivation studies [87]. In a subsequent study Miller et al. [95] reported that highly purified preparations of the TTX binding protein, with a specific activity of greater than 2000 pmol TTX bound per mg protein, contain only a single large polypeptide (mol wt = 260,000). This large polypeptide is analogous to the large α subunit of the purified rat brain sodium channel and to the large subunit of the muscle sodium channel. There is no evidence that the eel channel contains any smaller subunits, and recent reconstitution studies suggest that the single 260,000 dalton polypeptide from eel represents a fully active sodium channel [119, 120].

Norman et al. [110], using *Tityus serrulatus* scorpion toxin to label the sodium channel, have also isolated the sodium channel from eel electroplax. The purified *Tityus* toxin binding activity contained a single large polypeptide (mol wt = 270,000), presumably identical to the TTX binding component isolated by Agnew et al. [2, 3, 95]. This result suggested that the 260,000 polypeptide from eel contains the binding sites for both TTX and the beta scorpion toxins.

Polypeptide Composition of the Sodium Channel from Chick Heart

Lombet and Lazdunski [90] have purified the sodium channel from chick cardiac sarcolemma, using *Tityus serrulatus* toxin and a TTX derivative to la-

bel the solubilized channel. The purified channel, which had a specific activity of about 1200 pmol TTX bound per mg protein, consisted of a single large polypeptide with a molecular weight of 230,000 to 270,000. The subunit compositions of the sodium channels isolated from electric eel, rat brain, rat skeletal muscle, and chick cardiac muscle are summarized in Table 2.

Table 2. Subunit composition of sodium channels from electric eel, rat brain, rat skeletal muscle, and chick cardiac muscle

Tissue	Subunit composition	References
Electric eel	mol wt = 260,000	[3, 95, 105]
Rat brain	mol wt (native channel) = 316,000 mol wt (subunits) = 260,000 39,000 37,000	[62, 63]
Rat skeletal muscle	mol wt (native channel) = 314,00 mol wt (subunits) = 260,000 45,000 38,000	[9]
Chick cardiac muscle	mol wt = 260,000	[90]

Reconstitution of the Purified Sodium Channel

When the sodium channel is solubilized from the membrane, it retains STX and TTX binding activity, but it does not bind the alkaloid neurotoxins or the alpha scorpion toxins. In the absence of an intact membrane, Na^+ transport through the channel cannot be used to measure channel function. High-affinity reversible STX and TTX binding have been used to identify the solubilized sodium channel and to develop the purification protocols described [2, 3, 9, 10, 62, 63]. More recently, the sodium channel has been purified by prelabeling the channel with *Tityus serrulatus* toxin, a beta scorpion toxin with an extremely slow dissociation rate, prior to solubilization [12, 90, 110]. However, in all of these studies specific TTX (STX) binding activity has been the primary criterion for estimating the purity of the channel. In the initial purification studies, the polypeptides which comigrated with TTX binding activity were referred to as the "TTX-binding component" or the "STX receptor" of the sodium channel, because it was not known whether the isolated subunits actually represented the entire sodium channel, or whether they represented only the TTX/STX receptor site on the channel. In order to prove that the purified components represented the entire sodium channel, it was necessary to show that complete sodium channel function could be recovered when the isolated polypeptides were reconstituted into an intact membrane.

Toxin-stimulated ion transport through the sodium channel has been reconstituted from crude preparations in which lobster nerve membrane fragments [149] or cholate extracts of brain membrane [58, 92] were incorporated into lipid vesicles. Tamkun and Catterall [137] successfully reconstituted toxin binding and action at three neurotoxin binding sites, as well as toxin-stimulated sodium transport, from unpurified cholate-solubilized rat brain sodium channel. Although the sodium channel used in these studies was not purified, this series of experiments demonstrated that sodium channel function was not irreversibly denatured when the channel was solubilized from the membrane.

Partially purified sodium channel preparations from rat muscle [151] and rat brain [134] have been reconstituted by adding excess phospholipid to the solubilized channel preparation, then removing the detergent by adsorption to polystyrene beads. This procedure yields fairly large (100 to 200 nm diameter) unilamellar vesicles with sufficient internal volume to permit the measurement of radioisotope uptake and efflux by a simple biochemical assay.

The partially purified rat skeletal muscle sodium channel, reconstituted into egg phosphatidylcholine, transports $^{22}Na^+$ in response to alkaloid toxin activation [151]. The concentrations of batrachotoxin and veratridine required for half-maximal stimulation of $^{22}Na^+$ uptake are comparable to those determined for synaptosomes [138] and neuroblastoma cells [25]. Labeled STX binds to a single high-affinity site on the reconstituted sarcolemmal channel, and STX blocks toxin-activated $^{22}Na^+$ flux through the channel. In a more recent study Tanaka et al. [140], using a rapid quenched-flow technique, showed that the reconstituted sarcolemmal sodium channel transports cations with the same selectivity that is characteristic of the channel in intact cells.

Talvenheimo et al. [134] have shown that partially purified rat brain sodium channels, reconstituted into egg phosphotidylcholine vesicles, transport $^{22}Na^+$ in response to veratridine or batrachotoxin activation and bind STX with a dissociation constant of 3 nM, identical to the K_d for STX binding to synaptosomes. The temperature stability of STX binding is also completely restored following reconstitution of the sodium channel into vesicles. Toxin-stimulated $^{22}Na^+$ flux through the brain channel is blocked by TTX and by the local anesthetic tetracaine. In subsequent experiments using highly purified sodium channels, Tamkun et al. [139] demonstrated that the reconstituted rat brain sodium channel also displays normal cation selectivity (Fig. 3). The permeability ratio $P_{Na}/P_{Rb}/P_{Cs}$ calculated for the toxin-activated reconstituted

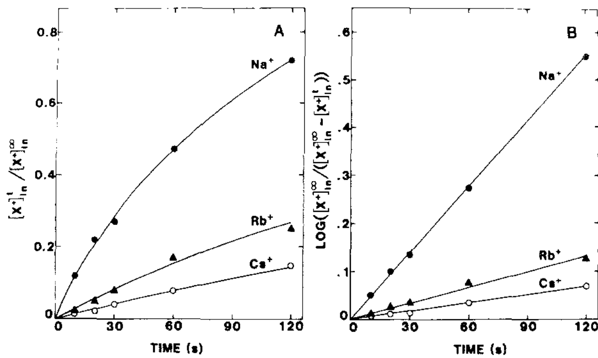

Fig. 3. Cation selectivity of the purified reconstituted sodium channel from rat brain. $^{22}Na^+$, $^{86}Rb^+$, and $^{137}Cs^+$ uptake were measured in the presence of 100 μM veratridine. Samples in 67.5 mM Na_2SO_4 were diluted into Na^+-free medium containing the radioactive tracer at time zero, incubated at 37°C for the indicated time, then assayed for isotope uptake by a cation exchange resin assay [134, 139]. (*A*) The ratio of internal isotope concentration at time *t*, ($[X^+]^t_{in}$), to the maximum steady-state isotope concentration predicted for the measured membrane potential ($[X^+]^\infty_{in}$) is plotted versus time. (*B*) The uptake data from (*A*) are plotted on semilogarithmic coordinates. (Reprinted from Tamkun et al. [139])

channel agrees well with the values determined for the toxin-activated channel in frog node of Ranvier [82] and in neuroblastoma cells [75]. These reconstitution studies demonstrated that the STX receptors isolated from both rat brain and rat skeletal muscle contain the protein components required for alkaloid toxin action, ion transport, and local anesthetic binding, in addition to the TTX/STX binding site [134–136, 139, 140, 151].

Tamkun et al. [139] have also provided quantitative evidence that the highly purified STX receptor from rat brain, consisting of three subunits, represents a completely functional sodium channel. By using rat brain STX receptor preparations estimated to be greater than 90% pure, and reconstituting at a low channel-per-vesicle ratio, it was estimated that 30 to 70% of the reconstituted STX receptors exhibited the properties of native sodium channels, including cation-selective transport, activation by veratridine, and block by TTX, STX and tetracaine. Moreover, the highly purified brain sodium channel also binds the alpha scorpion toxin LqTx when reconstituted into an appropriate lipid environment [47, 48, 139]. These experiments demonstrated that the α, $\beta 1$, and $\beta 2$ polypeptides in purified rat brain STX receptor preparations are sufficient for the reconstitution of sodium channel function, since the observed activity could not be attributed to minor contaminants in the purified preparations.

TTX-sensitive neurotoxin-activated ion transport and heat-stable TTX binding activity have also been reconstituted from the partially purified eel electroplax sodium channel [119]. $^{22}Na^+$ transport through the reconstituted eel channel is blocked by TTX and by local anesthetics. Since the preparation consists primarily of a single 260,000 dalton polypeptide, these results suggest that the alkaloid toxin binding site, the local anesthetic binding site, the ion-translocating pore, and the TTX binding site all reside on a single polypeptide.

The biochemical assays used to assess the function of the reconstituted sodium channel have yielded substantial information regarding the functional state of the purified channel. However, in order to measure the voltage-dependent behavior of the purified channel on a physiological time-scale, electrophysiological techniques are required. Two different approaches have been used. The purified rat brain sodium channel has been incorporated into planar lipid bilayers [61, 65, 66], while the purified eel sodium channel has been incorporated into vesicles which permit patch-clamp recording [120].

Rosenberg et al. [120] recorded single-channel events from vesicles containing purified eel sodium channels. Channel openings were observed in the presence and in the absence of batrachotoxin. Although the TTX-sensitivity and voltage dependence of these events were not rigorously tested, the ion selectivity, the magnitude of the single-channel conductances, and the channel mean open times were consistent with the single-channel properties reported for sodium channels from other cell types [7, 72, 73, 132]. However, the frequency of successful recordings of single sodium channels by this method is low.

The incorporation of partially purified rat brain sodium channels into planar lipid bilayers was first reported by Hanke et al. [61], who recorded two types of channel event. The first type of channel event exhibited a conductance comparable to values reported for the synaptosomal sodium channel [85], and was selective for Na^+. However, this channel type also displayed bursting behavior, which is not characteristic of sodium channels, and its sensitivity to TTX was not thoroughly tested. The second type of channel event described by Hanke et al. [61] had a much larger conductance than any value reported for sodium channels in intact cells, exhibited little selectivity for Na^+, and was not blocked rapidly by TTX. Moreover, both classes of channels were activated by hyperpolarization, in contrast to native sodium channels. Although Hanke et al. suggest that this anomalous behavior may be due to the bilayer composition, it is clear that the channel events they observed cannot be attributed to sodium channels without further investigation.

Hartshorne et al. [66], using a method similar to that of Krueger et al. [85], have successfully incorporated purified rat brain sodium channels into planar lipid bilayers and characterized the neurotoxin sensitivity, ion selectivity, and voltage dependence of the purified sodium channel under voltage-clamp conditions. The opening of batrochotoxin-activated purified sodium channels was strongly voltage dependent. Hartshorne et al. [66] also demonstrated that the purified sodium channels are reversibly blocked by low concentrations of TTX, exhibiting the same voltage dependence for TTX block that has been observed for native rat brain sodium channels in planar bilayers [53]. Opening and closing of single purified sodium channels, and rapid block by TTX, are illustrated in Fig. 4. The purified sodium channel displayed a single-channel conductance and cation selectivity that agree with values determined for the native sodium channel [76, 85]. These results indicated that the purified rat brain sodium channel contains a functional voltage-sensing mechanism in addition to the alkaloid toxin binding site, TTX/STX binding site, scorpion toxin binding site, and ion-conducting pore.

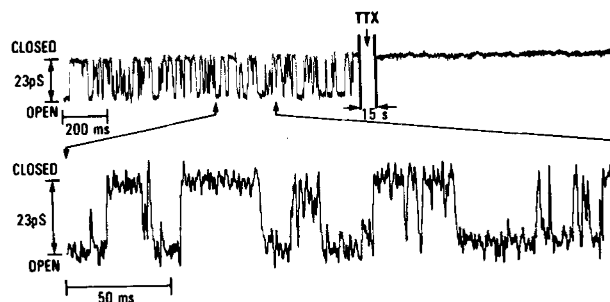

Fig. 4. Opening and closing of purified rat brain sodium channels reconstituted into a planar lipid bilayer. Sodium channels purified from rat brain were incorporated into planar lipid bilayers formed from a mixture of phosphatidylethanolamine and phosphatidylcholine. Single sodium channel currents, activated by 2 μM batrachotoxin, were recorded at −70 mV in the presence of a 0.5 M/0.2 M NaCl gradient. Channel opening was abolished by 3 μM TTX [63, 64]. (Reprinted from Hartshorne et al. [65])

Chemical Analysis of the Purified Sodium Channel

The solubilized sodium channels from rat sarcolemma [10], rat brain [62], and eel [97] bind to lectin affinity columns, indicating that the channel isolated from all three sources is glycosylated. Labeled lectins bind to the large subunit of the sarcolemmal sodium channel, but not to the smaller subunits separated on SDS-polyacrylamide gels [9]. In contrast, labeled wheat germ agglutinin binds to all three subunits of the brain sodium channel on SDS-polyacrylamide gels,[2] suggesting that a portion of each subunit is exposed at the extracellular surface. This finding also indicates that the small subunits observed in the rat muscle channel preparations are not analogous to the subunits of the isolated rat brain sodium channel.

Because it has been possible to purify milligram amounts of the eel sodium channel, the most extensive chemical analysis has been carried out on this preparation. Miller et al. [95] determined the amino acid and carbohydrate composition of the purified eel sodium channel. Perhaps the most striking result of this analysis is that the sodium channel contains 29.5% carbohydrate by weight, which may contribute to its unusually high electrophoretic mobility on SDS-polyacrylamide gels.

[2] D.J. Messner and W.A. Catterall, *unpublished results*.

Primary Sequence of the Purified Sodium Channel from Eel

Recently, Noda et al. [105] have deduced the entire amino-acid sequence of the electric eel sodium channel by cloning the DNA complementary to the mRNA encoding the channel, then sequencing the cloned cDNA. Noda et al. prepared a library of cDNA clones using poly(A)-RNA from eel electroplax as a template, and screened the expressed products of the library with antibodies to the purified eel channel. Using partial sequence information obtained from tryptic fragments of the purified sodium channel, Noda et al. [105] prepared a synthetic oligonucleotide probe containing a mixture of all possible cDNA sequences predicted for the pentapeptide Thr-Phe-Phe-Glu-Pro. The immunologically positive clones were isolated and tested for hybridization with the synthetic probe. One cDNA clone coding for a partial sequence of the channel was identified by hybridization with the oligonucleotide probe. The immunologically positive cDNA clones were also tested for hybridization with total eel RNA, which resulted in the identification of a second clone containing part of the cDNA sequence for the sodium channel protein. Together these cDNA clones were used to isolate additional cDNA clones encoding partial sequences for the sodium channel. A 7230-nucleotide sequence coding for the entire sodium channel protein was identified and sequenced using this approach.

The eel sodium channel protein contains 1820 amino-acid residues, for a total protein molecular weight of 208,321. Since carbohydrate accounts for 29.5% of the channel by weight, the predicted molecular weight of the glycosylated channel (295,491)

agrees very well with the previously reported molecular weight [3, 87, 95]. The amino-acid composition of the channel also agrees quite well with the composition reported by Miller et al. [95]. The channel contains ten asparagine residues that are potential N-glycosylation sites. Noda et al. [105] identified four repeated units within the sodium channel sequence, each consisting of approximately 260 amino acids, which exhibit extensive homology. They also analyzed the sequence for hydrophobic regions long enough to traverse the membrane, and reported that each of the four homologous units contains five such hydrophobic segments. Based on the analysis of hydrophobicity and predicted secondary structure, Noda et al. proposed that each of the four homologous units traverses the membrane four times, each unit contributing to the formation of the ion channel. Both the amino terminal and the carboxyl terminal are on the cytoplasmic side of the membrane in this model, a prediction that can be tested experimentally. The channel sequence contains four segments of negatively charged amino acids, including one highly unusual segment of nine sequential glutamic acids. Noda et al. have tentatively assigned the sequence of four negatively charged segments, located between the second and third homology units, to the cytoplasmic side of the membrane, and suggest that these segments may be involved in channel gating. Although this model may require modifications as more topological information becomes available from chemical and immunological studies, it is a very attractive one, and will provide the theoretical basis for future experimentation.

Future Directions for Sodium Channel Research

The ultimate objectives of ion channel purification are to define the molecular structure of the channel and to correlate structure with function. Using a combination of biophysical techniques, conventional purification methods, reconstitution studies, and recombinant DNA techniques, a substantial amount of progress has been made toward defining the molecular structure of the sodium channel. A large number of questions remain, however, regarding channel function at the molecular level. Information about the primary structure of the sodium channel will render some of these questions more accessible to experimental solutions.

The primary sequences of mammalian sodium channels are expected to be very similar, but not identical, to that of the electric eel sodium channel, because the channels share very similar biochemical and electrophysiological characteristics. Once this sequence information is obtained for the mammalian sodium channel, it will be possible to compare objectively the compositions of sodium channels from different tissues, as well as from different species. The immunological differences that have been observed [54, 152] among sodium channels isolated from different sources can probably be attributed at least in part to differences in the primary structure of the channels.

Using the technique of site-directed mutagenesis, in which single amino-acid substitutions at defined locations in the polypeptide chain are generated, the function of specific regions of the channel polypeptides can be defined. This approach will be useful for determining which parts of the molecule are involved in voltage-dependent gating, ion selectivity, activation and inactivation. Neurotoxin and drug binding sites on the channel can be identified by specific labeling studies in conjunction with sequencing data. Antibodies directed against specific peptide sequences can provide information about the location of these sequences with respect to the membrane. In addition to the mapping of specific functions to each segment of the sodium channel protein, the processes of channel biosynthesis, post-translational processing, and regulation of channel synthesis can be explored more easily using the information about channel structure that has emerged from purification studies. As more specific antibodies directed against defined portions of the sodium channel become available, along with probes for the mRNA encoding the channel, rapid progress can be expected in these areas.

Purification of the Voltage-Sensitive Calcium Channel

Voltage-sensitive calcium channels play a critical role in neurotransmitter release, generation of the cardiac action potential and excitation-contraction coupling [for reviews *see* 60, 84, 93, 145]. Biochemical characterization of the calcium channel has been facilitated by the development of an important group of drugs that block calcium channels. Using these drugs, a drug receptor site associated with the voltage-dependent calcium channel has recently been substantially purified.

Calcium channel blockers or "calcium antagonists" are a diverse group of compounds that act at two separate sites on the voltage-dependent calcium channel [98, 121, 155]. The dihydropyridines, which include nitrendipine and nifedipine, bind to a high-affinity site associated with the channel and can either block [34, 86, 144] or activate the channel [55, 128]. Verapamil and diltiazem bind to a second

site with lower affinity. Both verapamil and diltiazem block ion flux through the channel, but they have different allosteric effects on dihydropyridine binding. Verapamil allosterically inhibits labeled nitrendipine binding, while diltiazem enhances nitrendipine binding. The sites at which these two classes of drug act have been termed the "calcium antagonist receptor" [5, 86].

The approaches used to identify and isolate the polypeptide components of the calcium channel are very similar to those used for the sodium channel. The specific binding of labeled dihydropyridines, in particular ^{3}H-nitrendipine, has provided an assay for identifying the calcium channel in membranes and in solubilized preparations. The calcium antagonist receptor has been successfully solubilized from rat brain [38], rabbit skeletal muscle transverse tubule membranes [19, 20, 39], and from guinea pig skeletal muscle [56]. Radiation inactivation studies of the nitrendipine binding site in intact membranes indicate that it has a molecular weight of 278,000 in smooth muscle [148], 185,000 to 210,000 in skeletal muscle and in brain [49, 109].

Solubilization of the Calcium Antagonist Receptor from Brain

The calcium antagonist receptor from rat brain has been prelabeled with ^{3}H-nitrendipine, which dissociates very slowly at 4°C, and solubilized with digitonin [38]. The rate of ^{3}H-nitrendipine dissociation from the solubilized prelabeled receptor is enhanced by verapamil and slowed by diltiazem, indicating that the soluble receptor contains both the nitrendipine and the verapamil/diltiazem binding sites [38]. The soluble receptor binds specifically to wheat germ agglutinin, indicating that the calcium antagonist receptor, like the sodium channel, is a glycoprotein.

Although the calcium antagonist receptor can be solubilized in an active form from brain membranes, brain is a relatively poor starting material for purification of the receptor. Rat brain membranes contain 80 fmol of nitrendipine binding sites per mg membrane protein [38], about one fortieth of the concentration of voltage-sensitive sodium channels in rat brain. The molecular weight of the calcium antagonist receptor, estimated by radiation inactivation experiments, is approximately 210,000. If one mole of receptor binds one mole of nitrendipine, the specific activity of a pure preparation will be 4760 pmol nitrendipine bound per mg of protein, corresponding to a 60,000-fold purification from rat brain. By contrast, transverse tubule (T-tubule) membranes from rabbit skeletal muscle provide a very rich source of the calcium antagonist receptor, containing about 10 to 20 pmol of nitrendipine binding sites per mg protein [39, 56, 83]. Some investigators have reported concentrations as high as 50 pmol of nitrendipine binding sites per mg protein in T-tubule membrane preparations [2, 57]. Because T-tubule membranes contain a significantly higher concentration of the calcium antagonist receptor than any other tissue, T-tubule membranes have been used as the starting material for the initial purification studies described below.

Solubilization of the Calcium Antagonist Receptor from Skeletal Muscle

Glossmann and Ferry [56] have solubilized the calcium antagonist receptor from guinea pig skeletal muscle with the detergent CHAPS. The solubilized receptor retained reversible high-affinity dihydropyridine binding, which could be allosterically modified by diltiazem. In addition, the solubilized receptor retained stereospecific selectivity for optical isomers of D-600 (a calcium antagonist related to verapamil) and diltiazem. A purification of 17- to 40-fold was achieved by lectin affinity chromatography, consistent with identification of the receptor as a glycoprotein.

The calcium antagonist receptor from skeletal muscle T-tubule membranes has also been solubilized in digitonin and purified 330-fold by Curtis and Catterall [39]. After four purification steps, the receptor was estimated to be 41% pure with respect to specific nitrendipine binding activity. Three polypeptides, separated by SDS-polyacrylamide gel electrophoresis, comigrated with ^{3}H-nitrendipine binding activity throughout the purification: the α subunit (mol wt = 160,000), β subunit (mol wt = 53,000), and the γ subunit (mol wt = 32,000). After reduction with dithiothreitol, the apparent molecular weight of the α subunit on SDS-polyacrylamide gels shifted to 135,000, and the molecular weights of the β and γ subunits shifted to 50,000 and 33,000, respectively. No additional subunits were detected on gels following the reduction of disulfide bonds, suggesting that the shift in the apparent molecular weight of the subunit is due to the reduction of intramolecular disulfide bonds. It was concluded from these results that the calcium antagonist receptor from rabbit T-tubule membranes consists of three noncovalently associated subunits. The total molecular weight of a 1 : 1 : 1 complex of the α, β γ subunits would be consistent with the molecular weight of 210,000 determined by target analysis.

Borsotto et al. [19] have solubilized the calcium anatogonist receptor from T-tubule membranes,

Table 3. Molecular size of the calcium antagonist receptor associated with the voltage-dependent calcium channel

Membrane source	Molecular weight by target size analysis	Subunits identified by affinity labeling	Subunits identified by purification
Skeletal muscle T-tubules	178,000 [49]	145,000 [51]	130,000 50,000 [39]
	210,000 [109]	36,000 [83]	32,000 142,000 33,000 [20] 32,000
Cardiac sarcolemma	—	32,000 [23] 42,000 [74]	—
Brain	185,000 [50] 210,000 [109]	—	—

and purified the receptor approximately 10-fold in CHAPS [20]. Nitrendipine binding to the soluble receptor was inhibited by verapamil and enhanced by diltiazem, indicating that the coupling between the dihydropyridine binding site and the verapamil/diltiazem binding site remained intact, as reported by Glossmann and Ferry [56] and Curtis and Catterall [39]. The subunit composition of the partially purified calcium antagonist receptor, analyzed by SDS-polyacrylamide gel electrophoresis, was similar to that described by Curtis and Catterall [39], consisting of a polypeptide with a molecular weight of 142,000, and two smaller polypeptides with molecular weights of 32,000 and 33,000. No subunit corresponding to the β subunit (mol wt 50,000) identified by Curtis and Catterall was observed.

The polypeptide composition of the calcium antagonist receptor of the calcium channel from T-tubule membranes, determined by partial purification of dihydropyridine binding activity, is consistent with the molecular size determined by target size analysis, and with the results of covalent labeling experiments (Table 3). Ferry et al. [49] labeled a peptide of 145,000 with an arylazide dihydropyridine derivative in guinea pig skeletal muscle, which may correspond to the large subunit identified by Curtis and Catterall [39] and Borsotto et al. [20]. Similarly, a peptide with a molecular weight of 32,000 has been labeled by direct photoactivation of nitrendipine in cardiac membranes [23] and by an isothiocyanate derivative of nitrendipine [74]. This is consistent with the presence of a peptide of this size in partially purified calcium antagonist receptor preparations.

Phosphorylation of the Calcium Antagonist Receptor from Skeletal Muscle

Although little is known about the functions of the calcium antagonist receptor subunits, Curtis and Catterall [40] have shown that the β subunit associated with the partially purified receptor is selectively phosphorylated by cAMP-dependent protein kinase in T-tubule membranes. This is a particularly interesting finding because there is substantial evidence that calcium channel activity in cardiac cells is modulated directly or indirectly by cAMP-dependent protein phosphorylation [21, 111, 113, 114, 143, 146]. There is also evidence in perfused snail neurons that the maintenance of calcium currents may depend on cAMP-dependent phosphorylation [44, 45]. The results of Curtis and Catterall suggest that the β subunit of the calcium antagonist receptor may in fact be a regulatory subunit involved in the regulation of calcium channel function by cAMP-dependent phosphorylation.

It is not yet known whether the purified calcium antagonist receptor represents the entire voltage-sensitive calcium channel. There are several lines of evidence suggesting that the calcium antagonist receptor is either part of, or closely associated with, the calcium channel. First, drugs that bind to the calcium antagonist receptor either block ion permeation through the channel or activate the channel. Second, inhibition of the calcium channel by nitrendipine, verapamil and diltiazem is antagonized by the permeant cations Ca^{2+} and Ba^{2+} [86], suggesting that the drug binding sites are directly coupled to a cation binding site on the channel. Moreover, high-affinity dihydropyridine binding is directly modulated by divalent cations [18, 59, 91], providing additional evidence that the calcium antagonist receptor is coupled to divalent cation binding sites on the channel.

In smooth muscle [121] and in pheochromocytoma cells [142], the dissociation constant for labeled nitrendipine binding, and the total number of binding sites, agree well with the K_i for nitrendipine binding and the estimated number of calcium channels, respectively. In other tissues, such as cardiac muscle, the concentrations of nitrendipine required

to inhibit the calcium channel are much larger than the concentrations required to saturate the high-affinity nitrendipine binding sites [96, 99], leading to some question whether the high-affinity nitrendipine binding sites necessarily represent calcium channels. Bean [14] has suggested that nitrendipine inhibition of the calcium channel may depend on the functional state of the channel. In the heart, nitrendipine appears to act selectively on the inactivated state [14], providing a possible explanation for the observed discrepancies between labeled nitrendipine binding affinity in cardiac membrane preparations, where the channel is likely to be in the inactivated state, and the concentrations required for inhibition of calcium channel function in intact cells [42, 129]. It should be clear that the subunit composition of the calcium channel can be unambiguously defined only by reconstituting calcium channel function from completely purified subunits. Using the techniques that have been developed to reconstitute the voltage-dependent sodium channel, it will be possible to determine whether the calcium antagonist receptor is, in fact, a voltage-dependent calcium channel.

Genetic Approaches to the Isolation of Potassium Channels

Until recently the only practical approach to isolating ion channels was to use a specific high-affinity ligand as a label for the channel protein in membranes and in detergent-solubilized preparations. Even when such specific ligands have been available, as for the sodium channel, this approach has had disadvantages. With few exceptions, ion channels are present in low densities in most tissues, so that a very high degree of purification is needed to isolate the channel from other membrane proteins. Methods for solubilizing the channel proteins without irreversibly denaturing the ligand binding activity, and assays for the solubilized binding activity, have to be developed on an empirical basis. Similarly, methods for removing the detergent from the purified channel and incorporating the channel into phospholipid membranes must be devised in order to measure physiological channel functions.

With the exception of calcium-activated potassium channels [77, 94], no specific high-affinity ligands for any class of potassium channel have been identified. A completely different approach, a combination of genetic techniques and electrophysiology, is being used to isolate and clone the genes for ion channels for which there are no suitable biochemical probes. This approach has been most extensively developed for identifying the gene for the A channel, a fast transient potassium channel, in *Drosophila melanogaster* [124–126, 141, 153, 154].

Identification of the Shaker Locus in *Drosophila*

The locus of the A channel gene was identified by genetic analysis of mutant flies, which were isolated on the basis of a behavioral phenotype [80]. Flies with mutations in this locus shake when exposed to ether anesthesia. Jan and Jan [79] demonstrated that transmitter release at the neuromuscular junction in flies with mutations mapped to this particular locus, designated the "Shaker" locus, is prolonged due to prolongation of the action potential. The mutation was mimicked in wild-type flies by 4-aminopyridine, which blocks the A channel selectively, suggesting the defect was related to A channel function [80, 124, 126].

Salkoff [124, 125, 127] subsequently analyzed the ionic currents in Shaker mutants by intracellular recording and showed that the A current is either altered or missing in these mutants, while other ionic currents are unaffected. Three types of Shaker mutant have been isolated: those in which A currents are completely absent, those in which the biophysical characteristics of the current are altered. and a third type in which the A current is biophysically normal, but the amount of current is considerably diminished compared to wild-type. All of these mutations have been mapped genetically to the same locus [125]. The types of mutation which have been identified, along with several other lines of evidence, suggest that the Shaker locus contains a structural gene for the A channel. Other ionic currents are not altered in Shaker mutants, as might be expected if the mutations affected a general membrane property [80, 124, 126, 127]. Heterozygotes containing both mutant and wild-type genes appear to synthesize some normal A channels and some mutant channels [126], suggesting the defect is not in the biosynthetic pathway for the channel, although this possibility cannot be completely ruled out.

Cloning the Shaker Gene Product

The Shaker gene can be identified and cloned by tagging the gene with a transposable element of known sequence, a technique described by Bingham et al. [17]. Transposable elements (transposons) are DNA fragments capable of inserting themselves into the genome, frequently causing mutations at the site of insertion [88, 123]. A specific transposon of known sequence, designated the P factor, is particularly useful for these experiments, because its frequency of transposition in *Drosophila* can be increased by mating certain strains of flies [46, 122]. This increases the probability of incorporating the P factor into the Shaker locus and obtaining a transposon-induced mutation that can be iden-

tified by its phenotype. DNA fragments containing the "tagged" Shaker gene can be identified by hybridization with labeled P factor, and the DNA on either side of the inserted P factor can be isolated. Then the P factor-containing DNA that maps to the Shaker locus can be used to hybridize to the normal Shaker gene in wild-type flies that do not contain P factors.

Jan et al. [78] have successfully isolated P factor-induced Shaker mutants in which the mutation in the Shaker gene can be attributed to incorporation of the transposable element. They have outlined two approaches to demonstrating that the Shaker locus actually codes for the A channel protein. If the A channel consists of a single polypeptide subunit, the cloned Shaker DNA can be used to isolate the appropriate mRNA for the A channel. By injecting the isolated mRNA into oocytes, it will be possible to demonstrate the synthesis of A channels directly using intracellular recording techniques. However, if the A channel is a multi-subunit protein and not all the subunits are encoded by the Shaker locus (which is unlikely because all the Shaker mutations identified have mapped to the same location), the problem is more complex. The sequence for the subunit coded by the Shaker locus would have to be identified by hybridization to a specific mRNA required for expression of A channels. Antibodies directed against the product of this sequence could then be used to isolate the multi-subunit complex.

Conclusion

It should be emphasized that the problem of isolating a gene for which the gene product has been defined solely by genetic criteria is not trivial, but this approach is potentially very powerful. Not only does it provide a means for determining the sequence and structure of ion channels for which there are no biochemical probes, but it also provides a system in which specific mutations in the channel gene can be correlated with changes in channel function. Although this approach is limited to organisms that are readily manipulated by genetic methods, the results of these studies should be widely applicable. The biophysical properties of ion channels are generally similar from one species to another, suggesting that the structures of the channels have been highly conserved. The combination of biochemical, biophysical and genetic techniques will undoubtedly be exploited more fully in future studies of ion channel structure and function.

I would like to thank Dr. William A. Catterall, Dr. Richard E. Weiss and Benson M. Curtis for their helpful suggestions.

References

1. Agnew, W.S. 1984. *Annu. Rev. Physiol.* **46:**517–530
2. Agnew, W.S., Levinson, S.R., Brabson, J.S., Raftery, M.A. 1978. *Proc. Natl. Sci. USA* **75:**2606–2611
3. Agnew, W.S., Moore, A.C., Levinson, S.R., Raftery, M.A. 1980. *Biochem. Biophys. Res. Commun.* **92:**860–866
4. Agnew, W.S., Raftery, M.A. 1979. *Biochemistry* **10:**1912–1919
5. Akaike, N., Brown, A.M., Nishi, K., Tsuda, Y. 1981. *Br. J. Pharmacol.* **74:**87–95
6. Albuquerque, E.X., Daly, J.W. 1976. *In:* Receptors and Recognition. P. Cuatrecasas, editor. Vol. 1, pp. 299–336. Chapman and Hall, London
7. Aldrich, R.W., Corey, D.P., Stevens, C.F. 1983. *Nature (London)* **306:**436–441
8. Anholt, R., Lindstrom, J., Montal, M. 1983. *In:* Enzymes of Biological Membranes. A. Martonosi, editor. pp. 335–401. Plenum, New York
9. Barchi, R.L. 1983. *J. Neurochem.* **40:**1377–1385
10. Barchi, R.L., Cohen, S.A., Murphy, L.E. 1980. *Proc. Natl. Acad. Sci. USA* **77:**1306–1310
11. Barhanin, J., Giglio, J.R., Leopold, P., Schmid, A., Sampaio, S.V., Lazdunski, M. 1982. *J. Biol. Chem.* **252:**12553–12558
12. Barhanin, J., Pauron, D., Lombet, A., Norman, R.I., Vijverberg, H.P.M., Giglio, J.R., Lazdunski, M. 1983. *EMBO J.* **2:**915–920
13. Bernard, E.A., Beeson, D., Bilbe, G., Brown, D.A., Constanti; A., Conti-Tronconi, B.M., Dolly, J.O., Dunn, S.M.J., Mehraban, F., Richards, B.M., Smart, T.G. 1983. *Cold Spring Harbor Symp. Quant. Biol.* **48:**109–124
14. Bean, B.P. 1984. *Proc. Natl. Acad. Sci. USA* **81:**6388–6392
15. Beneski, D.A., Catterall, W.A. 1980. *Proc. Natl. Acad. Sci. USA* **77:**639–642
16. Benzer, T.I., Raftery, M.A. 1973. *Biochem. Biophys. Res. Commun.* **51:**939–944
17. Bingham, P.M., Levis, R., Rubin, G.M. 1981. *Cell* **25:**693–704
18. Bolger, G.I., Gengo, P., Klockowski, R., Luchowski, E., Siegel, H., Janis, R.A., Triggle, A.M., Triggle, D.J. 1983. *J. Pharmacol. Exp. Ther.* **225:**291–309
19. Borsotto, M., Barhanin, J., Norman, R.I., Lazdunski, M. 1984. *Biochem. Biophys. Res. Commun.* **122:**1357–1366
20. Borsotto, M., Norman, R.I., Fosset, M., Lazdunski, M. 1984. *Eur. J. Biochem.* **14:**449–455
21. Brum, G., Flockerzi, V., Hofmann, F., Osterrieder, W., Trautwein, W. 1983. *Pfluegers Arch.* **398:**147–154
22. Cahalan, M.D. 1975. *J. Physiol. (London)* **244:**511–534
23. Campbell, K.P., Lipshutz, G.M., Denney, G.H. 1984. *J. Biol. Chem.* **259:**5384–5387
24. Casadei, J.M., Gordon, R.D., Lampson, L.A., Schotland, D.L., Barchi, R.L. 1984. *Proc. Natl. Acad. Sci. USA* **81:**6227–6231
25. Catterall, W.A. 1975. *Proc. Natl. Acad. Sci. USA* **71:**1782–1786
26. Catterall, W.A. 1975. *J. Biol. Chem.* **250:**4053–4059
27. Catterall, W.A. 1977. *J. Biol. Chem.* **252:**8660–8668
28. Catterall, W.A. 1979. *J. Gen. Physiol.* **74:**375–392
29. Catterall, W.A. 1980. *Annu. Rev. Pharmacol. Toxicol.* **20:**15–43
30. Catterall, W.A. 1984. *Science* **223:**653–661
31. Catterall, W.A., Morrow, C.S., Hartshorne, R.P. 1979. *J. Biol. Chem.* **254:**11379–11387

32. Catterall, W.A., Nirenberg, M. 1973. *Proc. Natl. Acad. Sci. USA* **70**:3759–3763
33. Catterall, W.A., Ray, R., Morrow, C.S. 1976. *Proc. Natl. Acad. Sci. USA* **73**:2682–2686
34. Cauvin, C., Loutzenhiser, R., Breemen, C. van 1983. *Annu. Rev. Pharmacol. Toxicol.* **23**:373–396
35. Claudio, T., Ballivet, M., Patrick, J., Heinemann, S. 1983. *Proc. Natl. Acad. Sci. USA* **80**:1111–1115
36. Conti-Tronconi, B.M., Raftery, M.A. 1982. *Annu. Rev. Biochem.* **51**:491–530
37. Courand, F., Jover, E., Dubois, J.M., Rochat, H. 1982. *Toxicology* **20**:9–16
38. Curtis, B.M., Catterall, W.A. 1983. *J. Biol. Chem.* **258**:7280–7283
39. Curtis B.M., Catterall, W.A. 1984. *Biochemistry* **23**:2113–2118
40. Curtis, B.M., Catterall, W.A. 1985. *Proc. Natl. Acad. Sci. USA (in press)*
41. Darbon, H., Jover, E., Courand, F., Rochat, H. 1983. *Biochem. Biophys. Res. Commun.* **115**:415–422
42. DePover, A., Grupp, I.L., Grupp, G., Schwartz, A. 1983. *Biochem. Biophys. Res. Commun.* **114**:922–929
43. Devillers-Theiry, A., Giraudat, J., Bentaboulet, M., Changeux, J.-P. 1983. *Proc. Natl. Acad. Sci. USA* **80**:2067–2071
44. Doroshenko, P.A., Kostyuk, P.G., Martynyuk, A.E. 1982. *Neuroscience* **7**:2125–2134
45. Doroshenko, P.A., Kostyuk, P.G., Martynyuk, A.E., Kursky, M.D., Vorobetz, Z.D. 1984. *Neuroscience* **11**:263–267
46. Engels, W.R. 1981. *Cold Spring Harbor Symp. Quant. Biol.* **45**:561–565
47. Feller, D.J., Talvenheimo, J.A., Catterall, W.A. 1984. *Soc. Neurosci.* **10**:255.4
48. Feller, D.J., Talvenheimo, J.A., Catterall, W.A. 1985. *J. Biol. Chem. (in press)*
49. Ferry, D.R., Goll, A., Glossmann, H. 1983. *EMBO J.* **2**:1729–1732
50. Ferry, D.R., Goll, A., Glossmann, H. 1983. *Naunyn-Schmied. Arch. Pharmacol.* **323**:292–297
51. Ferry, D.R., Rombusch, M., Goll, A., Glossmann, H. 1984. *FEBS Lett.* **168**:42–52
52. Fosset, M., Jaimovich, E., Delpont, E., Lazdunski, M. 1983. *J. Biol. Chem.* **258**:6086–6092
53. French, R.J., Worley, J.F., Krueger, B.K. 1984. *Biophys. J.* **45**:301–310
54. Fritz, L.C., Brockes, J.P. 1983. *J. Neurosci.* **3**:2300–2309
55. Garcia, A.G., Sala, F., Reig, J.A., Viniegra, S., Frias, J., Fonteriz, R., Gandia, L. 1984. *Nature (London)* **309**: 69–71
56. Glossmann, H., Ferry, D.R. 1983. *Naunyn-Schmied. Arch. Pharmacol.* **323**:279–291
57. Glossmann, H., Ferry, D.R., Boschek, C.B. 1983. *Naunyn-Schmied. Arch. Pharmacol.* **323**:1–11
58. Goldin, S.M., Rhoden, V., Hess, E.J. 1980. *Proc. Natl. Acad. Sci. USA* **77**:6884–6888
59. Gould, R.J., Murphy, K.M.M., Snyder, S.H. 1982. *Proc. Natl. Acad. Sci. USA* **79**:3656–3660
60. Hagiwara, S., Byerly, L. 1981. *Annu. Rev. Neurosci.* **4**:69–125
61. Hanke, W., Boheim, G., Barhanin, J., Pauron, D., Lazdunski, M. 1984. *EMBO J.* **3**:509–515
62. Hartshorne, R.P., Catterall, W.A. 1981. *Proc. Natl. Acad. Sci. USA* **78**:4620–4624
63. Hartshorne, R.P., Catterall, W.A. 1984. *J. Biol. Chem.* **259**:1667–1675
64. Hartshorne, R.P., Coppersmith, J.C., Catterall, W.A. 1980. *J. Biol. Chem.* **255**:10572–10575
65. Hartshorne, R.P., Keller, B.U., Talvenheimo, J.A., Catterall, W.A., Montal, M. 1984. *Soc. Neurosci.* **10**:255.5
66. Hartshorne, R.P., Keller, B.U., Talvenheimo, J.A. Catterall, W.A., Montal, M. 1985. *Proc. Natl. Acad. Sci. USA* **82**:240–244
67. Harthshorne, R.P., Messner, D.J., Coppersmith, J.C., Catterall, W.A. 1982. *J. Biol. Chem.* **255**:13888–13891
68. Henderson, R., Wang, J.H. 1972. *Biochemistry* **11**:4565–4569
69. Hille, B. 1968. *J. Gen. Physiol.* **51**:199–219
70. Hille, B. 1984. Ionic Channels of Excitable Membranes. Sinauer Associates, Sunderland, Massachusetts
71. Hodgkin, A.L., Huxley, A.F. 1952. *J. Physiol. (London)* **117**:500–544
72. Horn, R., Patlack, J., Stevens, C.F. 1981. *Biophys. J.* **36**:321–327
73. Horn, R., Vandenberg, C.A., Lange, K. 1984. *Biophys. J.* **45**:323–335
74. Horne, P., Triggle, D.J., Venter, J.C. 1984. *Biochem. Biophys. Res. Commun.* **121**:890–898
75. Huang, L.-Y.M., Catterall, W.A., Ehrenstein, G. 1979. *J. Gen. Physiol.* **73**:839–854
76. Huang, L.-Y.M., Moran, N., Ehrenstein, G. 1984. *Biophys. J.* **45**:313–322
77. Hugues, M., Romey, G., Duval, D., Vincent, J.P., Lazdunski, J. 1982. *Proc. Natl. Acad. Sci. USA* **79**:1308–1312
78. Jan, L.Y., Barbel, S., Timpe, L., Laffer, C., Salkoff, L., O'Farrell, P., Jan, Y.N. 1983. *Cold Spring Harbor Symp. Quant. Biol.* **48**:233–245
79. Jan, Y.N., Jan, L.Y. 1980. *In:* Insect Neurobiology and Pesticide Action. F.E. Rickett, editor. p. 161. The Society of Chemical Industry, London
80. Jan, Y.N., Jan, L.Y., Dennis, M.J. 1977. *Proc. R. Soc. London. B* **198**:87–108
81. Karlin, A. 1980. *In:* The Cell Surface and Neuronal Function. C.W. Cotman et al., editors. p. 191. Elsevier/North Holland, Amsterdam
82. Khodorov, B.I. 1978. *In:* Membrane Transport Processes. D.C. Tosteson, Y.A. Ovchinnikov, and R. Latorre, editors. Vol. 2, pp. 153–174. Raven, New York
83. Kirley, T.L., Schwartz, A. 1984. *Biochem. Biophys. Res. Commun.* **123**:41–49
84. Kostyuk, P.G. 1981. *Biochim. Biophys. Acta* **650**:128–150
85. Krueger, B.K., Worley, J.F., French, R.J. 1983. *Nature (London)* **303**:172–175
86. Lee, K.S., Tsien, R.W. 1983. *Nature (London)* **302**:790–794
87. Levinson, S.R., Ellory, J.C. 1972. *Nature, New Biol.* **245**:122–123
88. Lewin, B. 1983. Genes. pp. 609–631. John Wiley and Sons, New York
89. Lindstrom, J., Anholt, R., Einarson, B., Engel, A., Osame, M., Montal M. 1980. *J. Biol. Chem.* **255**:8340–8350
90. Lombet, A., Lazdunski, M. 1984. *Eur. J. Biochem.* **141**:651–660
91. Luchowski, E.M., Yousif, F., Triggle, D.J., Maurer, S.C., Sarmiento, J.G., Janis, R.A. 1984. *J. Pharmacol. Exp. Ther.* **230**:607–613
92. Malysheva, M.K., Lishko, V.K., Chagovetz, A.M. 1980. *Biochim. Biophys. Acta* **602**, 70–77
93. McDonald, T.F. 1982. *Annu. Rev. Physiol.* **44**:425–434
94. Miller, C., Moczydlowski, E., Latorre, R., Phillips, M. 1985. *Nature (London)* **313**:316–318

95. Miller, J.A., Agnew, W.S., Levinson, S.R. 1983. *Biochemistry* **24:**462–470
96. Miller, R.J., Freedman, S.B. 1984. *Life Sci.* **34:**1205–1221
97. Moore, A.C., Agnew, W.S., Raftery, M.A. 1982. *Biochemistry* **24:**6212–6220
98. Murphy, K.M.M., Gould, R.J., Largent, B.L., Snyder, S.H. 1983. *Proc. Natl. Acad. Sci. USA* **80:**860–864
99. Nachshen, D.A., Blaustein, M.P. 1979. *Mol. Pharmacol.* **16:**579–586
100. Nakamura, Y., Nakajima, S., Grundfest, H. 1965. *J. Gen. Physiol.* **42:**965–974
101. Narahashi, T. 1974. *Physiol. Rev.* **54:**813–889
102. Narahashi, T., Moore, J.W., Scott, W.R. 1964. *J. Gen. Physiol.* **42:**965–974
103. Nelson, N., Anholt, R., Lindstrom, J., Montal, M. 1980. *Proc. Natl. Acad. Sci. USA* **77:**3057–3061
104. Noda, M., Furutani, Y., Takahashi, H., Toyosato, M., Tanabe, T., Shimizu, S., Kikyotani, S., Kayano, T., Hirose, T., Inayama, S., Numa, S. 1983. *Nature (London)* **305:**818–823
105. Noda, M., Shimizu, S., Tanabe, T., Takai, T., Kayano, T., Ikeda, T., Takahashi, H., Nakayama, H., Kanaoka, Y., Minamino, N., Kangawa, K., Matsuo, H., Raftery, M.A., Hirose, T., Inayama, S., Hayashida, H., Miyata, T., Numa, S. 1984. *Nature (London)* **312:**121–127
106. Noda, M., Takahashi, H., Tanabe, T., Toyosato, M., Furutani, Y., Hirose, T., Asai, M., Inayama, S., Miyata, T., Numa, S. 1982. *Nature (London)* **299:**793–797
107. Noda, M., Takahashi, H., Tanabe, T., Toyosato, M., Kikyotani, S., Furutani, Y., Hirose, T., Asai, M., Takashima, H., Inayama, S., Miyata, T., Numa, S. 1983. *Nature (London)* **302:**528–532
108. Noda, M., Takahashi, H., Tanabe, T., Toyosato, M., Kikyotani, S., Hirose, T., Asai, M., Takashima, H., Inayama, S., Miyata, T., Numa, S. 1983. *Nature (London)* **301:**251–255
109. Norman, R.I., Borsotto, M., Fosset, M., Lazdunski, M., Ellory, J.G. 1983. *Biochem. Biophys. Res. Commun.* **111:**878–883
110. Norman, R.I., Schmid, A., Lombet, A., Barhanin, J., Lazdunski, M., 1983. *Proc. Natl. Acad. Sci. USA* **80:**4164–4168
111. Osterrieder, W., Brum, G., Hescheler, J., Trautwein, W., Flockerzi, V., Hofmann, F. 1982. *Nature (London)* **298:**576–578
112. Popot, J.-L., Changeux, J.-P. 1984. *Physiol. Rev.* **64:**1162–1239
113. Reuter, H. 1974. *J. Physiol. (London)* **242:**429–451
114. Reuter, H., Scholz, H. 1977. *J. Physiol. (London)* **264:**49–62
115. Ritchie, J.M., Rogart, R.B., Strichartz, G.R. 1976. *J. Physiol. (London)* **261:**477–494
116. Rochat, H., Rochat, C., Kupeyan, C., Miranda, R., Lissitzky, S., Edman, P. 1970. *FEBS Lett.* **10:**349–351
117. Romey, G., Abita, J.P., Schweitz, H., Wunderer, G., Lazdunski, M. 1976. *Proc. Natl. Acad. Sci. USA* **73:**4055–4059
118. Romey, G., Chicheportiche, R., Lazdunski, M., Rochat, H., Miranda, R., Lissitzky, S. 1975. *Biochem. Biophys. Res. Commun.* **64:**115–121
119. Rosenberg, R.L., Tomiko, S.A., Agnew, W.S. 1984. *Proc. Natl. Acad. Sci. USA* **81:**1239–1243
120. Rosenberg, R.L., Tomiko, S.A., Agnew, W.S. 1984. *Proc. Natl. Acad. Sci. USA* **81:**5594–5598
121. Rosenberger, L.B., Triggle, D.J. 1977. *In:* Calcium and Drug Action. G.B. Weiss, editor. pp. 3–31. Plenum, New York
122. Rubin, G.M. 1982. *Cell* **29:**987–994
123. Rubin, G.M. 1983. *In:* Mobile Genetic Elements. J.A. Shapiro, editor. pp. 329–362. Academic, New York
124. Salkoff, L. 1983. *Nature (London)* **302:**249–251
125. Salkoff, L. 1983. *Cold Spring Harbor Symp. Quant. Biol.* **48:**221–231
126. Salkoff, L., Wyman, R. 1981. *Nature (London)* **293:**228–230
127. Salkoff, L., Wyman, R. 1983. *J. Physiol. (London)* **337:**687–709
128. Schramm, M., Thomas, G., Towart, R., Franckowiak, G. 1983. *Nature (London)* **309:**535–537
129. Schwartz, A., Triggle, D.J. 1984. *Annu. Rev. Med.* **35:**325–339
130. Sharkey, R.G., Beneski, D.A., Catterall, W.A. 1985. *Biochemistry* **23:**6078–6086
131. Sigel, E., Stephenson, F.A., Mamalaki, C., Barnard, E.A. 1983. *J. Biol. Chem.* **258:**6965–6971
132. Sigworth, F.J., Neher, E. 1980. *Nature (London)* **282:**447–449
133. Sumikawa, K., Houghton, M., Smith, J.C., Bell, L., Richards, B.M., Bernard, E.A. 1982. *Nucleic Acids Res.* **10:**5809–5822
134. Talvenheimo, J.A., Tamkun, M.M., Catterall, W.A. 1982. *J. Biol. Chem.* **257:**11868–11871
135. Talvenheimo, J.A., Tamkun, M.M., Hartshorne, R.P., Messner, D.J., Sharkey, R.G., Costa, M.R.C., Catterall, W.A. 1983. *Cold Spring Harbor Symp. Quant. Biol.* **48:**155–164
136. Talvenheimo, J.A., Tamkun, M.M., Messner, D.J., Hartshorne, R.P., Sharkey, R.G., Catterall, W.A. 1984. *Biophys. J.* **45:**37–40
137. Tamkun, M.M., Catterall, W.A. 1981. *J. Biol. Chem.* **256:**11457–11463
138. Tamkun, M.M., Catterall, W.A. 1981. *Mol. Pharmacol.* **19:**78–86
139. Tamkun, M.M., Talvenheimo, J.A., Catterall, W.A. 1984. *J. Biol. Chem.* **259:**1676–1688
140. Tanaka, J.C., Eccleston, J.F., Barchi, R.L. 1983. *J. Biol. Chem.* **258:**7519–7526
141. Tanouye, M.A., Ferrus, A., Fujita, S.C. 1981. *Proc. Natl. Acad. Sci. USA* **78:**6548–6552
142. Toll, L. 1982. *J. Biol. Chem.* **257:**13189–13192
143. Trautwein, W., Taneguchi, J., Noma, A. 1982. *Pfluegers Arch.* **392:**307–314
144. Triggle, D.J. 1982. Calcium Blockers: Mechansisms of Action and Clinical Applications. S.F. Flain, and R. Zelis, editors. pp. 121–134. Urban and Schwarzenberg, Baltimore
145. Tsien, R.W. 1983. *Annu. Rev. Physiol.* **45:**341–358
146. Tsien, R.W., Weingart, R. 1976. *J. Physiol. (London)* **260:**117–141
147. Ulbricht, W. 1969. *Ergeb. Physiol. Biol. Chem. Exp. Pharmakol.* **61:**18–71
148. Venter, J.C., Fraser, C.M., Schaber, J.S., Jung, C.Y., Bolger, G., Triggle, D.J. 1983. *J. Biol. Chem.* **258:**9344–9348
149. Villegas, R., Villegas, G.M., Barnola, F.V., Racker, E. 1979. *In:* Advances in Cytopharmacology. B. Ceccarelli and F. Clementi, editors. Vol. 3, pp. 373–385. Raven, New York

150. Wang, G.K., Strichartz, G.R., 1982. *Biophys. J.* **40**:175–179
151. Weigele, J., Barchi, R. 1982. *Proc. Natl. Acad. Sci. USA* **79**:3651–3655
152. Wollner, D.A., Catterall, W.A. 1985. *Brain Res.* **331**:145–149
153. Wu, C.-F., Ganetzky, B. 1984. *Biophys. J.* **45**:77–78
154. Wu, C.-F., Ganetzky, B., Haugland, F.N., Liu, A.-X. 1983. *Science* **220**:1076–1078
155. Yamamura, H.I., Shoemaker, H., Boles, R.G., Roeske, W.R. 1980. *Biochem. Biophys. Res. Commun.* **108**:640–646

Received 15 March 1985